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Evaluation of IFN-γ effects on apoptosis and gene expression in neuroblastoma—Preclinical studies

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Abstract

Loss of caspase-8 expression and resistance to cytotoxic agents occurs frequently in late stage neuroblastoma (NB). Interferon-γ (IFN-γ) induces caspase-8 in NB cells, sensitizing them to death receptor mediated apoptosis. This study characterizes the kinetics of this phenomenon and examines the effects of IFN-γ on global gene expression to determine whether IFN-γ responses are achievable at physiologically relevant doses and to define the biological effects of this cytokine. Here we examine the IFN-γ responses of 16 NB cell lines. A single <5-min exposure to IFN-γ (0.5 ng/ml) induced caspase-8 expression in all non-expressing cell lines and in 3/6 cell lines which already expressed high caspase-8. This increase in caspase-8 proteins was observed within 16 h and persisted for up to 9 days. Furthermore, IFN-γ pretreatment of NB cells increased doxorubicin-induced apoptosis nearly 3-fold. Microarray analysis was used to identify additional genes involved in proliferation, signaling and apoptosis whose expression was modulated via IFN-γ. Altered expression of these genes should further enhance the responsiveness of NB cells to chemotherapeutics. Thus, the use of IFN-γ to sensitize NB cells to cytotoxic agents represents an attractive therapeutic strategy and warrants further investigation.

Keywords: Interferon-γ; *MYCN*; Neuroblastoma; Caspase-8; Apoptosis; Microarray

1. Introduction

Apoptosis in multicellular organisms is a tightly regulated process required for normal growth, development, and cellular specialization. Defective expression of proteins and aberrant function of constituents in the apoptotic cascade have been implicated in oncogenesis, tumor progression, and treatment resistance [1–4]. Two distinct arms of the apoptotic pathway predominate in chemotherapeutic drug-induced cell death: the death receptor (or extrinsic) pathway and the mitochondrial (or intrinsic) pathway. Stimulation of death receptors of the tumor necrosis factor (TNF) family, CD95/APO-1/Fas, DR4, and DR5, convert quiescent procaspase-8 and procaspase-10 molecules to their active forms, caspase-8 and caspase-10. These initiator caspases then activate other downstream proapoptotic caspases and factors resulting in cell death. One downstream target of caspase-8 is Bid, a proapoptotic bcl-2 family member. Upon activation, Bid facilitates cytochrome-c release from the mitochondria, thereby activating the mitochondrial arm of the intrinsic pathway. A distinct set of chemotherapeutic drugs and other cellular stresses initiate apoptosis at the mitochondrial level, which results in the release of cytochrome-C, Apaf-1, and caspase-9 into the cytosol [1–4]. These mitochondrial proteins associate with calcium ions and form the apoptosome, which ultimately activates caspase-3. Caspase-3 activates multiple caspases (downstream and upstream) including caspase-8. When activated in this manner, caspase-8 functions in an amplification loop with caspase-3 to enhance and accelerate the apoptotic response [5–7].

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Decreased caspase-8 protein, frequently in association with N-myc amplification, has been observed in advanced stage or high risk neuroblastoma (NB), large cell medulloblastoma, alveolar rhabdomyosarcoma, and small cell lung carcinoma, all of which are tumors of neural or neuroectodermal origin [2,8–13]. Decreased caspase-8 expression has been correlated with methylation of both CASP-8 alleles in a region located at the exon3/intron3 boundary and not with methylation of promoter sequences or with gene mutations or deletions such as those commonly seen in tumor suppressor genes [13]. Despite this correlation, a cause effect relationship between the methylation of these sites and caspase-8 expression has not been established to date. However, NB cells with absent or diminished caspase-8 expression demonstrated marked resistance to death-inducing stimuli such as TNFá, TRAIL, cycloheximide, and some chemotherapeutic agents (e.g., doxorubicin), all of which activate the extrinsic arm of the apoptotic pathway in NB cells in vitro [13–15].

Induction of endogenous caspase-8 expression with DNA methyltransferase inhibitors (DNMTi), such as 5-azacytidine, or following transduction of tumor cells with caspase-8-expressing retroviruses reconstituted a functional extrinsic apoptotic pathway, resensitizing the tumor cells to cytotoxic agents and significantly enhancing apoptosis in vitro [13–15]. Although theoretically possible, practical application of DNMTi, caspase-8-expressing retroviruses, or both in vivo is only now being examined [16]. Phase 1 clinical trials are currently in progress to determine the feasibility, side effects, toxicity and usefulness of DNMTi in neuroblastoma patients. Importantly, recent studies indicate that exposure to DNMTi may be associated with tumor induction in mice [17,18], though extrapolation of these data to humans remains controversial [19].

Recently, interferon-γ (IFN-γ) has been shown to induce caspase-8 expression in NB cell lines, irrespective of the methylation status of the *CASP8* gene, rendering the cells sensitive to cytotoxic stimuli [20–25]. However, the previous studies have not determined the threshold dose required for caspase-8 expression or examined the kinetics of γ-IFN mediated caspase-8 induction in detail. Therefore it is unclear whether this response could reasonably be achieved in vivo. Furthermore, although it has been established that IFN-γ influences the expression of numerous genes [26–28] many of which inhibition of cell growth and/or stimulation of cell differentiation [26–28], the effects of IFN-γ on gene expression in NB have not been characterized in detail. Here we evaluate the concentration dependence of IFN-γ associated apoptotic sensitization and determine the time course and duration of this process in detail. We confirm that treatment with IFN-γ and doxorubicin together amplifies the apoptotic responses of NB cells to these agents, and establish that sequential administration of IFN-γ followed by doxorubicin is necessary to achieve maximal cell death. Finally, we describe the overall effect of IFN-γ on gene expression in neuroblastoma using microarray analysis to identify changes in mRNA expression, thereby allowing us to determine how this cytokine influences NB cell growth and chemoresponsiveness. The preclinical studies presented here suggest that IFN-γ could be used as a therapeutic agent in NB patients and that further studies using xenograph models are warranted.

2. Materials and methods

2.1. Cell Lines and culture methods

The human NB cell lines used in these studies have been described in detail elsewhere [13,29]. Cells were maintained in culture in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 0.1% gentamicin Invitrogen (Carlsbad, CA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Reagents

Doxorubicin, IFN-γ and 5-Aza-2′deoxycytidine were purchased from Sigma (St. Louis, MO). z-IETD-fmk, a caspase inhibitor with high specificity for caspase-8, was purchased from Enzyme System Products, Valeant Pharmaceuticals International (Costa Mesa, CA). All reagents were prepared and stored according to the manufacturer's instructions.

2.3. Treatment of cells

For caspase-8 expression studies, cells were plated at a density of 3.5×10^5 cells/60 mm dish, allowed to adhere to the culture dish, and then treated with IFN-γ (0.5–100 ng/ml) for different periods of time (5 min to 24 h) as indicated in the figures. In experiments lasting longer than 48 h the culture media was replaced with fresh media every 48 h. In studies using 5-aza-2′deoxycytidine and IFN-γ, the demethylating agent (1 or 5 μM) was administered every 24 h for 2 days. The cells were then treated with IFN-γ (100 ng/ml) for 120 min, washed with PBS, and incubated in fresh media for an additional 16 h before harvest. For studies involving both doxorubicin and IFN-γ, IFN-γ treatment preceded doxorubicin administration by 24 h. Doxorubicin (0.1 μg/ml) exposure was maintained for an additional 48 h before the cells were harvested for TUNEL analysis.

For the functional studies, cells were plated as previously described [13]. In the IFN-γ treatment group, NB cells were treated with IFN-γ (100 ng/ml) for 120 min. IFN-γ was then removed, and the cells were allowed to recover for 16 h before harvest for further analysis. In the studies with both doxorubicin and IFN-γ treatment, IFN-γ (100 ng/ml) was administered on day 1 as described above and doxorubicin (0.1 μg/ml) was applied on days 2 and 3. In experiments involving z-IETD-fmk (50 μM), the inhibitor was present in the culture medium for the duration of the experiment. Culture dishes from all treatment groups were harvested on day 4 and analyzed for apoptosis by TUNEL as described below.

2.4. Immunoblot analysis for caspase-8 expression

Immunoblots containing 25 to 50 μg of total cellular protein lysate were prepared as described previously [29]. Immunoblots were sequentially incubated with mouse anti-caspase-8 C15 monoclonal antibody (1/50) [30] or an anti-actin control antibody Santa Cruz Biotechnology Inc (Santa Cruz, CA) (1:1000) and a 1:4000 dilution of horseradish peroxidase-conjugated IgG goat anti-mouse secondary antibody Kierkegaard and Perry Laboratories (Gaithersburg, MD). ECL Western blotting detection reagents from GE Healthcare (Piscataway, NJ) were used to detect bound antibodies on the immunoblots.

2.5. Bisulfite Treatment and Methylation-Specific Polymerase Chain Reaction

Tumor cell genomic DNA (gDNA) was isolated with DNAzole (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's specifications. The final product was resuspended in 10 mM Tris–HCl (pH 7.5) and 0.1 mM EDTA and then quantified via standard spectrophotometric analysis.

The gDNA (1 μg) was bisulfite-modified by using the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to the manufacturer's

specifications. The modified gDNA was then used for methylation-specific polymerase chain reaction (MS-PCR) studies using the caspase-8 methylation primer sets previously described [13]. Reaction conditions included 4 mM $MgCl_2$, 20 nM each of the 5′ and 3′ primers, 10 μM dNTPs (2.5 μM of each nucleotide), and 0.5 μg of bisulfite-modified gDNA. All reactions were initially incubated at 95 °C for 10 min, and 0.5 μl Taq polymerase was added after 5 min. The MS-PCR using the unmethylated primer set included 32 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 45 s followed by a single cycle at 72 °C for 4 min; this reaction yielded a 322-bp fragment. Analysis using the methylated primer set included 35 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 45 s followed by a single cycle at 72 °C for 4 min; this reaction yielded a 321-bp fragment.

2.6. Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) analysis was performed as described previously using fluorochrome-labeled human *TNFR2* and centromeric chromosome 1p probes as controls, a P1 phage probe for human *CASP8*, and a cosmid probe containing human *MYCN* to determine whether the expression of *MYCN* was amplified in NB cells [13].

2.7. Analysis of apoptosis by TUNEL

In situ labeling of apoptosis-induced DNA strand breaks was carried out using an *In Situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Briefly, 3–4 slides containing a minimum of 700 cells were analyzed from 2 independent experiments. Cells were grown to 75% confluence on 60-mm culture plates. Cytospin preparations were made and the cells were fixed with paraformaldehyde and permeabilized with sodium citrate. TUNEL (terminal deoxinucleotidyltransferase-mediated dUTP-biotin nick-end-labeling) reagent was applied followed by Vectashield (4,6-diaminido-2 phenylindole) Vector Laboratories (Burlington, CA) application. Samples were analyzed for apoptotic events by visualization using indirect immunofluorescence microscopy. Intact cells displaying brightly stained nuclei with concurrent blebbing of the cell membrane were counted as apoptotic cells. The apoptotic index was determined by assessing the fraction of apoptotic cells in at least 8 to 10 high-powered fields on duplicate slide preparations; 700 to 800 cells were counted for each sample. Standard error of the mean was calculated for the data from three independent experiments.

2.8. RNase protection assay

RNA was isolated from the NB cell lines using TriReagent Invitrogen (Carlsbad, CA) per the manufacturer's instructions. Fifteen micrograms of total RNA was used for the RNAse protection analyses. These assays were performed using the hApoI and hApoIII RNase protection kits according to the manufacturer's directions BD Bioscience (San Diego, CA).

2.9. Microarray studies

Microarray analyses were conducted on the NB10, NB16, NB19, and NB1643 cells, all of which expressed wild-type p53. NB16 and NB1643 expressed caspase-8 and did not have amplified *MYCN*; NB10 and NB19 did not express caspase-8 and had amplified *MYCN*. Each cell line was treated with IFN-γ (100 μg/ml) for 2 h and then allowed to recover for 24 h prior to harvesting. RNA was obtained from control and IFN-γ-treated NB cells from three independent experiments using TRIzol reagent Invitrogen (Carlsbad, CA) as described by the manufacturer. The total RNA was further purified using the RNeasy Mini Kit Qiagen (Valencia, CA) to remove any residual genomic DNA.

For microarray analysis, five micrograms of total RNA from each sample was amplified using the MessageAmp RNA Amplification kit Ambion (Austin, TX). The amplified mRNA was then used to generate cDNA. Cye dye labeling was performed using an indirect, random hexamer-labeling method. Microarray analyses were performed according to MIAME, (minimum information about microarray experiments) standards. Human cDNA microarrays containing approximately 10,000 spots printed on each poly-lysine-coated slide were fabricated by the staff of the Functional Genomics Facility in the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital. All microarray experiments were performed in triplicate using separate biological samples. After hybridization, raw TIFF images from the scanner were uploaded to GenePix Pro version 4.1 (Axon Inc., Union City, CA) for analysis. Spot grids (.gal files) were manually fitted to the microarray images. The resulting GenePix files (.gpr files) were exported into Spotfire DecisionSite for Functional Genomics version 8.0 Spotfire Inc (Somerville, MA), and results were filtered to remove unreliable data on the basis of the signal-to-noise ratio [31]. The R statistical language-based Bioconductor version 1.9 (www.bioconductor.org) was used to perform further analyses, to normalize the dataset in an intensity-dependent manner [32], and to perform statistical analyses (i.e., *t*-test and ANOVA). Changes in the expression of genes were identified as statistically significant using volcano plots in Spotfire that took into account both the *P*-value and the fold change in expression. Changes in expression were counted as significant only if expression levels in all three experiments varied by at least 2-fold and the data had statistical P-values <0.05.

Using the Ingenuity Pathways Analysis Solution and Ingenuity Pathways Knowledge Base Ingenuity Systems (Mountain View, CA) software programs, several networks of gene interactions with significant network scores were identified. The network score (negative log of *P*-value) indicates the probability that a pathway can be formed through the chance assembly of its members. The networks were then joined on the basis of overlapping genes, and the most abundant functional categories were determined.

2.10. Real-time PCR

Quantitative real time PCR was used to further validate the microarray results. For these studies, RNA was extracted from control and IFN-γ treated cell using TRIzol reagent Invitrogen (Carlsbad, California) and residual DNA removed using the RNeasy Mini kit Qiagen (Valencia, CA). Purified RNAs (2 μg) were then used as template for cDNA synthesis using TaqMan Reverse Transcriptase Kit ABI Biosystems (Foster City, CA). Forty nanogram aliquots of each cDNA were used for Real-Time PCR to determine the expression levels of 18S rRNA, HLA-G, SOCS1, SOC3, and CASP4 using primers and conditions described previously [33–36]. All samples were run in triplicate and the experiment was repeated three times with independently isolated RNA. Gene expression levels were normalized to 18S using the comparative Ct method ($2^{-\Delta\Delta CT}$) as previously described [37].

3. Results

3.1. IFN-γ increases caspase-8 protein expression in NB cells without affecting CASP8 methylation

Since previous studies had focused on the effects of IFN-γ in caspase-8 deficient NB cell lines, we sought to determine the frequency of caspase-8 induction in a larger panel of cells and to determine whether caspase-8 expression could also be enhanced in cell lines that already expressed this protein. Caspase-8 expression was quantitated in 16 NB tumor cell lines by immunoblot analysis (Fig. 1A). All 10 NB cell lines with methylated *CASP8* alleles and absent/low expression of caspase-8 exhibited increased protein expression after γ-IFN treatment. IFN-γ exposure also increased caspase-8 expression in three of six cell lines which expressed caspase-8 prior to IFN treatment (Fig. 1A and Table 1). In accordance with the results from previous studies, IFN-γ treatment did not alter the status of *CASP8* methylation in any of these cell lines (Fig. 1B and [20,25]).

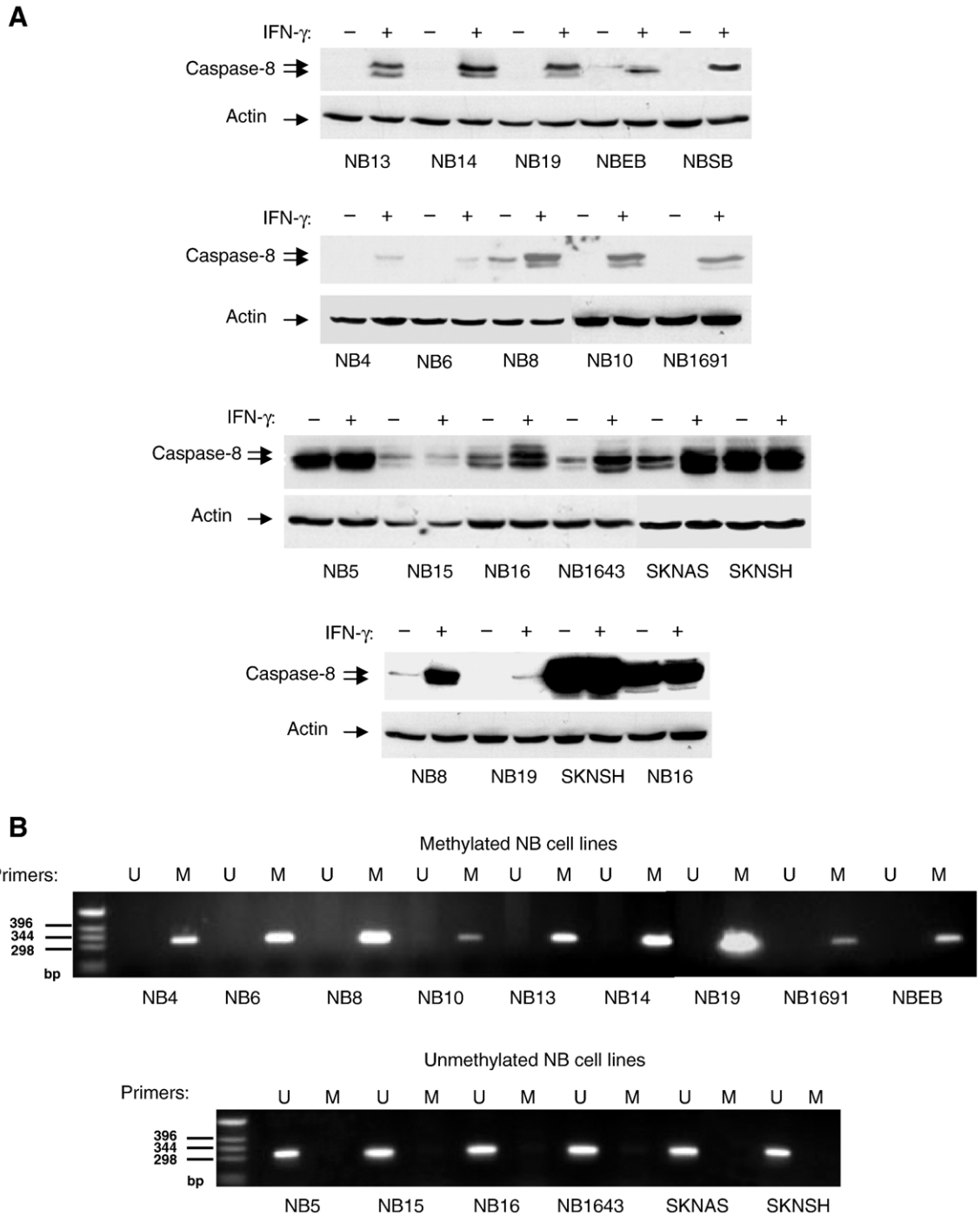

Fig. 1. Caspase-8 expression is enhanced by IFN-γ treatment without altering *CASP8* methylation status. (A) Immunoblot analysis of caspase-8 expression in NB cell lines before (−) and 16 h after (+) treatment with 100 ng/ml IFN-γ for 120 min. Each lane in the top two panels contains 50 μg of the indicated protein lysates, while panel 3 contains 30 μg of protein lysates from the caspase-8 expressing cell lines. To permit better comparison 50 μg of protein from the indicated cell lines with and without IFN-γ treatment were compared directly. (B) MS-PCR analysis of the indicated cells lines before and after treatment with IFN-γ. The primers used are indicated above each lane: the methylated primer set (M) produced a 321-bp product, and the unmethylated primer set (U) produced a 322-bp product. IFN-γ did not alter the methylation status of the *CASP8* alleles in any of the six representative NB cell lines.

3.2. Characterization of the exposure length and time course of IFN-γ-induced caspase-8 expression

To determine the duration of IFN-γ exposure necessary to induce caspase-8 protein expression, we exposed the cell lines to 100 ng/ml IFN-γ for various lengths of time ranging from 5 min to 48 h, washed the cells extensively with complete media and incubated in standard media for the remainder of the experiment. The IFN-γ treated cells were harvested 48 h after the onset of treatment and the protein lysates were examined for caspase-8 expression by immunoblot. This analysis revealed that a 5 min exposure to 100 ng/ml IFN-γ was sufficient to

Table 1
Characteristics of 16 human neuroblastoma (NB) cell lines that expressed normal *CASP8* alleles

Cell Line	TNFR2 [a] 1p36$^{-/-}$	MYCN amplification [b]	Caspase-8 Expression	CASP8 Methylation	IFN-γ dose/Caspase-8 expression [c]	Relative increase in Caspase-8 expression [d]
NB4	LOH	>20	−	+	2/100	3
NB5	LOH	2–5	+	−	0	3
NB6	LOH	>20	−	+	0.5/20	3
NB8	LOH	>20	−	+	0.5/5	3
NB10	LOH	>20	−	+	0.5/0.5	4
NB13	LOH	>20	−	+	0.5/5	1
NB14	LOH	>20	−	+	10/20	2
NB15	WT	>20	+	−	0	2
NB16	LOH	2–4	+	−	0.5/0.5	3
NB19	LOH	>20	−	+	10/10	3
NB1643	LOH	2–4	+	−	0.5/0.5	3
NB1691	LOH	>20	−	+	0.5/2	2
NBEB	LOH	>20	−	+	0.5/2	1
NBSB	LOH	>20	−	+	0.5/2	1
SKNAS	WT	2–4	+	−	0.5/0.5	4
SKNSH	LOH	2–4	+	−	0	4

[a] Fluorescent in situ hybridization (FISH) using a probe for *TNFR2* (tumor necrosis factor receptor 2) was used to determine whether1p36 was wild type (WT) or showed a loss of heterozygosity (LOH).
[b] MYCN copy number as determined by FISH.
[c] The minimum IFN-γ concentration (ng/ml) capable of inducing Caspase 8 expression/ IFN-γ concentration resulting in maximal caspase-8 expression.
[d] The relative level of caspase-8 protein after IFN-γ treatment on a scale of 0 (lowest) to 4 (highest).

induce caspase-8 expression in all responsive cell lines and that IFN-γ exposure beyond 30 min did not further enhance caspase-8 protein expression (Fig. 2A) and data not shown.

To determine the kinetics IFN-γ induction of caspase-8 protein and mRNA expression we measured caspase-8 mRNA and protein in cells harvested at varying times intervals after IFN-γ a two h IFN-γ treatment. RNase protection analysis revealed that caspase-8 mRNA expression began to increase approximately 10 h after IFN-γ treatment and reached a maximal level at 12 h (data not shown). These data are consistent with results from immunoblot studies that revealed the maximal level of caspase-8 protein expression was achieved 16 h after exposure to IFN-γ (Fig 2B).

To determine the duration of IFN-γ-mediated effects on caspase-8 expression, all 14 IFN-γ responsive neuroblastoma cell lines were treated with 50 to 100 ng/ml of IFN-γ for 120 min. The cells were then collected 1-9 days after the removal of IFN-γ for preparation of cellular lysates and immunoblot analysis. The results demonstrated that caspase-8 was up-regulated 1 day after treatment and remained elevated for 4 to 6 days (Fig. 2C). The level of caspase-8 returned to baseline 7–9 days post IFN-γ exposure (Fig 2C and data not shown). Finally, repeated IFN-γ treatment (100 ng/ml exposure for 2 h followed by a 16 h incubation period) demonstrated re-induction of caspase-8 protein expression at levels similar to those observed after the initial γ-IFN exposure (Fig. 2D).

3.3. Determination of IFN-γ dose required for caspase-8 induction

To define the dose of IFN-γ exposure necessary for caspase-8 protein induction, NB cells were treated with various doses IFN-γ (0.5–100 ng/ml) for 120 min followed by a 16 h culture period. Five of 10 cell lines showed increased caspase-8 expression after a low dose of IFN-γ (0.5 ng/ml), and all ten demonstrated substantial induction after treatment with 10 ng/ml IFN-γ (Fig. 2E). Interestingly, although both the minimal and maximal doses for caspase-8 induction varied for the different cell lines, induction of caspase-8 protein expression was initially IFN-γ concentration dependent but then reached a plateau and could not be enhanced further in any of the cell lines by increasing either the IFN-γ dose or the duration of treatment (see Table 1, column 6). The degree of caspase-8 protein induction varied across the cell lines tested, though overall maximum level of expression after IFN-γ treatment was considerably lower in the NB cell lines with low/absent caspase-8 genes than it was in untreated caspase-8 expressing NB cell lines (Fig. 1A, bottom panel). Preliminary experiments suggest it may be possible to further increase the level of caspase-8 expression in at least a subset of NB cells by sequential or combined treatment with demethylating agents such as 5-aza-2′deoxycytidine and IFN-γ (Fig. 2F). Similar data has also been obtained by other investigators [38].

3.4. IFN-γ-mediated restoration of sensitivity to apoptotic stimuli

To assess the functional integrity of the IFN-γ-induced caspase-8 protein and determine whether the level of protein expressed after IFN-γ treatment was sufficient for caspase-8-dependent apoptosis, we examined the effects of prior IFN-γ exposure on doxorubicin induced apoptosis. Although our studies demonstrate maximal up-regulation of caspase-8 expression with IFN-γ exposure of 20 ng/ml or less, an IFN-γ concentration of 100 ng/ml was chosen for the functional apoptosis studies to facilitate comparison with data reported in

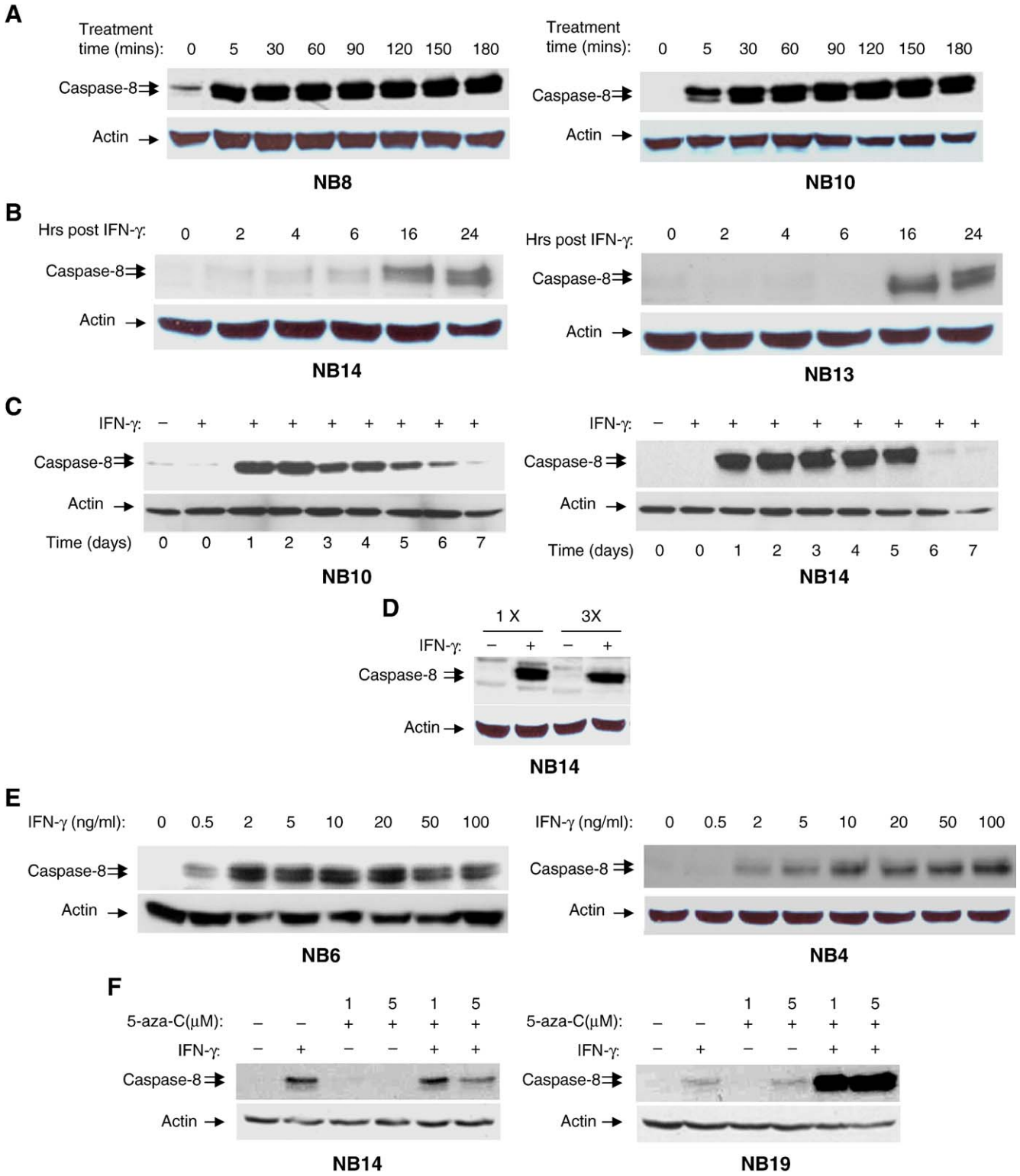

Fig. 2. Kinetics and time course of IFN-γ-induction caspase-8 in NB cells. (A) Immunoblot analysis of caspase-8 expression in NB8 and NB10 cell line. Cells were treated with 100 ng/ml IFN-γ for 0 to 180 min, and lysates were prepared 16 h later. (B) Maximal caspase-8 protein levels were observed in all NB cell lines within 16 h of IFN-γ treatment (100 ng/ml for 120 min). Results from 2 representative NB cell lines are shown. (C) Caspase-8 protein expression induced by IFN-γ (100 ng/ml for 120 min) persisted for 4 to 6 days. (+) indicates IFN-γ treated cells whereas (−) indicates untreated cells. (D) Repeated exposure (every 7 days) to 100 ng/ml of IFN-γ for 120 min did not alter the level of caspase-8 protein expression. Cells were harvested 16 h after the first and third IFN-γ exposure and blotted for caspase-8 and actin expression. (E) Analysis of IFN-γ concentration (ng/ml) required for induction of caspase-8 protein. Cells were treated for 120 min with the indicated concentration of INF-γ, wash and harvested 16 h later for immunoblot analysis. Data for the other cell lines are summarized in Table 1. (F) Effects of sequential treatment of NB cells with 5-aza-2′-deoxycytidine (1 or 5 μM as indicated) and IFN-γ (100 ng/ml) on NB14 and NB19. Enhanced caspase-8 expression was observed upon combination treatment for NB19 but not in NB14.

the literature. In these studies, we examined the effects of doxorubicin, IFN-γ, and z-IETD-fmk on apoptosis in 4 NB cell lines with *MYCN* amplification and low/absent caspase-8 protein expression under a variety of experimental conditions by TUNEL. IFN-γ induction of caspase-8 expression altered the sensitivity of these cells to doxorubicin, a chemotherapeutic drug that has been reported to initiate apoptosis predominantly through the extrinsic (i.e., Fas/CD95) arm of the apoptotic pathway in NB tumor cells [14]. Similar data were obtained upon treatment of the cell lines used in Fig. 3 with the IFN-γ dosage required for maximal caspase-8 protein production (data not shown).

The median basal level of apoptosis in untreated cells was 9.6% (range 1.1%–14.4%) (Fig. 3). No significant increase in the percentage of apoptotic cells was observed after treatment with either IFN-γ alone (median level of apoptosis 11.4%; range 8.9%–16.3%) or z-IETD-fmk alone (median 10.8%; range, 7%–21.1%). However, a dramatic increase in the level of apoptosis was observed in cells treated with both IFN-γ and doxorubicin (median 59%; range 40.5%–81.9%). Furthermore, this level of cell death was significantly greater than the sum of the percentages of apoptotic cells seen in cultures treated with either IFN-γ or doxorubicin alone, indicating that the sequential administration of IFN-γ and doxorubicin resulted in an amplified apoptotic response. The addition of z-IETD-fmk to cell cultures treated with IFN-γ and doxorubicin resulted in a substantial but not complete decrease in the apoptotic responses (Fig. 3). This result supports the involvement of caspase-8 in doxorubicin-induced cell death in NB cells. However, since z-IETD does not completely eliminate doxorubicin induced apoptosis, other caspases and/or proapoptotic proteins may also be induced by exposure to IFN-γ thereby increasing the apoptotic response of NB cells to treatment with chemotherapeutic agents. Indeed, the microarray data support this hypothesis.

3.5. IFN-γ-induced alterations in the expression of many genes that have potential roles in neuroblastoma tumorigenesis

To assess the effects of IFN-γ on global gene expression we used microarray analysis to compare mRNA expression in 4 NB cell lines (NB10, NB16, NB19, and NB1643) before and after IFN-γ exposure. NB10 and NB19 cells contained amplified *MYCN* alleles and, before IFN-γ treatment, exhibited very low caspase-8 expression. In contrast, NB16 and NB1643 cells expressed caspase-8, and their *MYCN* loci were not amplified. Alterations in RNA expression were validated by real time quantitative PCR using primers specific for HLA-G, SOCS1, SOCS3 and caspase 4. Data from this quantitative PCR analysis were similar to the microarray data in all cases. For example, microarray analysis of mRNA isolated from untreated and IFN-γ treated NB10 cells yielded an average increase in SOCS1 and caspase-4 expression of 13.4- and 3.2-fold respectively in the IFN-γ treated cells while real time PCR indicated a 12.1-fold in SOC1 expression and a 3.1-fold increase in caspase-4 expression. Similar data were obtained for the other cell lines with all four sets of primers (data not shown).

The microarray revealed substantial variation in gene expression levels between the cell lines, although the data from the three independent experiments were very similar for each cell line. However, the majority of the genes exhibiting significant changes in expression could be grouped into 5 somewhat overlapping functional pathways using the Ingenuity Pathways Analysis Solution and Knowledge Base software program. The first functional group contained sixty-five apoptosis-related genes which showed 2-fold or greater change in mRNA levels after treatment with IFN-γ ($P \leq 0.05$) in one or more cell lines in each of three independent experiments. Although there was inter-cell line variability in the IFN-γ mediated induction of some apoptotic genes, we were able to identify a set of genes whose expression is likely to be altered in

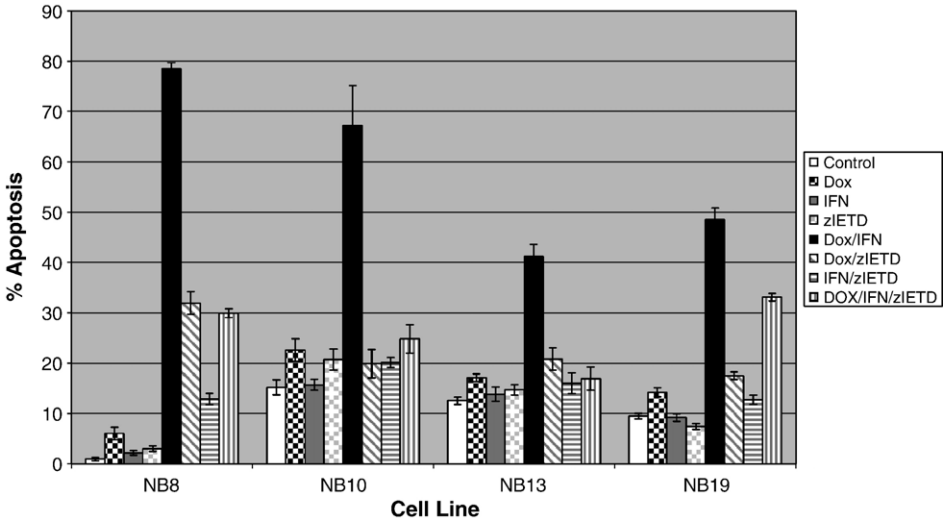

Fig. 3. Comparison of apoptosis in 5 NB cell lines. Apoptosis levels of NB cells which increase caspase-8 expression after IFN-γ treatment were assessed via TUNEL assay. Single-agent treatments included doxorubicin (dox; 0.1 μg/ml), IFN-γ (100 ng/ml) and z-IETD-fmk (z-IETD; 50 μM). Multi-agent treatments consisted of IFN-γ (100 ng/ml) followed by doxorubicin (0.1 μg/ml) alone or doxorubicin (0.1 μg/ml) plus z-IETD-fmk (50 μM). Error bars represent standard error of the mean from duplicate slides from 3 independent experiments.

most neuroblastoma cells lines, regardless of *MYCN* amplification or *CASP8* intron3/exon3 methylation status (Table 2). For example, three of the 4 cell lines showed increased *CASP4*, *CASP5, CASP7,* and *BCL-6* mRNA expression, and 2 of the 4 showed increased *CASP1* and *CARD4* (Table 2). Interestingly, *CASP8* mRNA expression was significantly increased in only the NB16 cell line using the criteria described above, although RNAse protection assays revealed increased RNA expression in all four cell lines and immunoblots showed a greater than 2-fold increase in caspase-8 protein expression in all 4 cell lines (Fig. 1 and data not shown). The expression of 58 genes associated with cell cycle regulation and 50 genes with roles in transcription or signal transduction was also altered in the various cell lines. Again, a small subset of genes involved in cell cycle (i.e., *CDKN1A, SOCS1, SSTR2* and *TYM*) and growth inhibition and differentiation (i.e., *IRF-1*, *SOCS3*, and *STAT-1*) showed increased expression, while those that promote cell division (i.e., *ARG2*, *RGS2*, and *SOCS2*) showed reduced expression in two or more cell lines (Table 2). Finally, we identified fifty immune response genes whose expression was effected by IFN-γ exposure in the various cell lines. A set of common genes with enhanced expression and functional role in promoting cellular differentiation and immune cell function (i.e., *CXCL11, HLA-B,* and *IL12A)* was observed this group.

Table 2
Microarray mRNA expression analysis

Function	NB16	NB1643	NB10	NB19
Apoptosis				
CASP4↑, CASP5↑, PLP1↑	X	X	X	
IRS1↑	X	X		X
CASP7↑, BCL6↑	X		X	X
TNFRSF1B↑	X		X	
CASP1↑, CARD4↑		X	X	
EVI1↓, IGFSBP4↑			X	X
CASP8↑,	X			
Cell cycle/proliferation				
SOCS1↑	X	X	X	X
CDKN1A↑	X		X	X
SSTR2↑	X	X		
TYMS↓		X		X
Signal transduction/transcription factors				
SOCS3↑	X	X	X	X
HLA-B↑, IRF1↑	X	X	X	
CXCL10↑	X	X		X
STAT1↑	X		X	
RGS2↓, SOCS2↓				X
IRF7↑		X		X
ARG2↓			X	X
Immune response				
HLA-G↑, LGALS3BP↑	X	X	X	X
CX3CL1↑, G1P2↑, HLA-B↑	X	X	X	
B2M↑	X		X	X
CXCL11↑	X	X		
IL12A↑	X		X	
TRIM22↑		X	X	

X, gene is expressed.
↑, expression is increased ≥ 2-fold, $p > 0.05$.
↓, expression is decreased ≤ 2-fold, $p > 0.05$.

4. Discussion

Neuroblastoma is the most common pediatric solid tumor that does not involve the central nervous system (CNS), constituting 20% of all pediatric non-CNS solid tumors in children aged 0 to 14 years [39,40]. At diagnosis, 80% of patients have advanced-stage (stages III or IV) or high-risk disease (e.g., *MYCN* amplified, chromosome 1p36.3 deletion, unfavorable histology). Historically, survival of these patients is less than 15% [39,40]. Although the implementation of high-dose myeloablative therapy with hematopoietic stem cell reconstitution has improved remission rates and prolonged event-free survival, long-term survival in patients with advanced-stage or high-risk neuroblastoma remains poor [41]. Relapsed disease in these patients is usually resistant to therapy [42]. Caspase-8 is not expressed in approximately 70% of NB cell lines and tumors with *MYCN* amplification and 1p36.3 LOH [13]. Absence of caspase-8 is also associated with resistance to cytotoxic drugs [13]. Lack of caspase-8 protein expression has been correlated with resistance to conventional chemotherapy in neuroblastoma and medulloblastoma in vitro and with clinical prognosis in medulloblastoma patients [8,15]. Recently, other investigators have shown that caspase-8 expression is induced in NB cells after prolonged IFN-γ exposure [20–25], a finding with potential clinical significance.

IFN-γ is a member of the IFN family of multifunctional cytokines with immune modulatory, antiproliferative, differentiation-promoting, and proapoptotic effects [26–28]. More than 200 genes are reported to be regulated by IFN-γ, and most, if not all, IFN-γ-dependent effects are mediated through IFN-receptors and the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway [26,27,43–46]. The changes in expression a heterogeneous group of cytostatic and apoptotic genes, in response to IFN-γ treatment appears to be determined by cellular context and prevailing microenvironment [26–28].

Although the mechanism is not completely understood, IFN-γ-mediated up-regulation of caspase-8 expression occurs through the JAK/STAT1 signal transduction pathway [20,25,46]. A key regulatory transcription factor in this process is interferon regulatory factor-1 (IRF-1) [20,25–28,43–46]. IRF-1 is a proapoptotic transcription factor induced by STAT1 that enhances STAT1-mediated transcriptional activation of caspase-8 [20,25,46]. Multiple caspase-8 isoforms have been identified, the majority of which function as proapoptotic mediators. Analyses of the human *CASP8* sequence have identified STAT1 and IRF-1 binding sites in the promoter region of the caspase-8 gene [24,25,46,47,49] within a hyper-methylated region of the exon3/intron3-boundary and throughout the caspase-8 genomic sequence. IFN-γ stimulation of NB cells with *CASP*8 methylation and low caspase-8 protein levels results in expression of two of the known proapoptotic caspase-8 isoforms, caspase-8a and caspase-8b, without altering intergenic methylation (Fig. 1 and [20,22,25]). Therefore, it remains unclear whether these sites and/or other interferon-sensitive response element sequences localized to other regions of the gene play any role in chromatin structure and/or caspase-8 expression.

Previous studies have indicated the potential usefulness of IFN-γ in the treatment of cancer, including neuroblastoma; however, these studies were performed under a single (or at most a few) experimental condition(s) [20–24,46]. Here we determined the dosage and kinetic properties of IFN-γ treatment and verified that the resulting induction of caspase-8 dramatically resensitized NB cells to chemotherapeutic drug-induced apoptosis. Our data indicate exposure to IFN-γ for 5 min is sufficient to induce caspase-8 expression in all of the NB cell lines with absent or low caspase-8 expression and to enhance caspase-8 expression in half (3/6) of cell lines that already expressed caspase-8. Moreover, increased caspase-8 mRNA expression was not detected until 12 h after IFN-γ exposure and maximal protein production was not observed until 16 h after stimulation suggesting that IFN-γ signals caspase-8 mRNA transcription and translation. Although low level caspase-8 expression was observed at INF-γ concentrations of 0.5–10 ng/ml, raising the dose of IFN-γ to 20–50 ng/ml resulted in maximal levels of caspase-8 protein. Caspase-8 expression then reached a plateau; no further increases in caspase-8 expression were observed beyond 20–50 ng/ml IFN-γ or with exposures beyond 30 min. Caspase-8 protein expression was maximally induced 16 h after treatment and was maintained for 4 to 6 days before gradually declining and ultimately disappearing approximately 5–9 days after treatment.

Although IFN-γ enhanced caspase-8 protein expression, microarray analysis demonstrated significant (>2-fold) up-regulation of *CASP8* in only 1 of the 4 cell lines at the transcriptional level. RNAse protection analysis revealed small, but significant, increases in caspase-8 transcripts in IFN-γ responsive cell lines. The likely explanation for these discrepant findings is the relative sensitivities of these two techniques and the 2-fold threshold used to identify significant differences by microarray [50]. Additionally, the relatively small increase in caspase-8 mRNA expression suggests that IFN-γ may also enhance caspase-8 protein expression at the post-transcriptional level.

The functional consequences of the IFN-γ effects in NB cells are dramatic and demonstrate that an amplified apoptotic response to doxorubicin in vitro can be obtained when IFN-γ and doxorubicin are administered sequentially. Similar results have been obtained by IFN-γ treatment of human myeloid leukemia, breast cancer and Ewing's sarcoma cells [48,51,52]. Our studies suggest that this apoptotic response is, at least in part, a consequence of caspase-8 activity because treatment of caspase-8-positive NB cell lines with z-IETD-fmk, a relatively specific caspase-8 inhibitor, decreased their sensitivity to doxorubicin-induced apoptosis. These observations support the assertion that in neuroblastoma, caspase-8 plays an important role in development of tumor cell resistance to some chemotherapeutic agents. However, the microarray analysis of IFN-γ-treated NB cell lines revealed up-regulation of transcripts for several other caspases and proapoptotic proteins, and down-regulation of several antiapoptotic and pro-proliferative transcripts. Indeed, increased expression of caspases-4, -5, and -7 might explain the observation that a small portion of the apoptotic activity we observed was not inactivated by treatment with z-IETD. This finding suggests that IFN-γ may also affect the responses of NB cells to chemotherapeutic agents that work via the mitochondrial (intrinsic) pathway.

The theoretical extrapolation of these data to in vivo systems suggests that physiologically achievable exposure times and systemic concentrations of IFN-γ are possible for treatment of patients with advanced-stage neuroblastoma. Information regarding IFN-γ therapy for pediatric malignancies is extremely limited; only 2 phase I clinical studies using IFN-γ alone have been reported to date [53,57]. In both studies, IFN-γ monotherapy was used as an immune modulator and was intended to augment or facilitate the host's antitumor response. Results from those studies were encouraging, but further studies have not yet been reported. Though extensive pharmacokinetic data are lacking, serum IFN-γ concentrations of 1.6–2.4 ng/ml were seen in patients receiving 325 μg/m^2 of IFN-γ [54]. Another study described successful administration of IFN-γ at doses of 0.8–2.7 μg/m^2 yielding serum IFN-γ concentrations of 5–20 ng/ml [55]. Of note, induction of caspase-8 was observed in IFN-γ responsive cell lines upon exposure to IFN-γ concentrations of 10 ng/ml or less, suggesting it should be possible to up-regulate caspase-8 expression in vivo [55–57]. Indeed, Thiele and coworkers [22] recently re-evaluated archived NB specimens obtained before and after IFN-γ treatment for caspase-8 expression and observed induction of caspase-8 after IFN-γ treatment in several of the biopsied tumors. This finding supports the conclusion that IFN-γ can induce caspase-8 expression in vivo.

Clinical trials of IFN-γ combined with conventional chemotherapeutic agents to treat adult patients with melanoma, ovarian, colon, or renal cell carcinoma have yielded promising results [58–65]. Based on our studies and the in vitro work from other laboratories and the analysis of archived biopsy samples from a previous small scale single agent IFN-γ clinical trial in human NB patients, we propose that IFN-γ be administered before cytotoxic therapy, not as an immune modulating agent but as a chemosensitizer to render tumors more responsive to conventional chemotherapy. As such, the temporal relationship and timing of IFN-γ and cytotoxic agent administration is important. IFN-γ treatment should precede that of conventional chemotherapeutic agents, allowing enough time for up-regulation of caspase-8 expression. Following IFN-γ exposure, the consequent enhanced caspase-8 expression appears to persist for several days. Our studies demonstrated enhanced cytotoxicity with doxorubicin treatment during this window post IFN-γ exposure in vitro. This sustained response should allow chemotherapy to be administered either by protracted continuous infusion or on a repetitive dosing schedule. Variations on this scheme have been proposed recently by other investigators based on their cell culture studies [22,66]. In these studies, these investigators evaluated the apoptotic sensitivity to TRAIL (i.e., TNF receptor apoptosis-inducing ligand) in tumor cells pretreated with chemotherapeutics (to enhance TRAIL2 receptor expression) and IFN-γ (to induce caspase-8 expression). These treatments induced caspase-8 protein expression in tumor cell lines and renewed their sensitivity to TRAIL-mediated apoptosis [22,66]. These results are similar to those of Debatin and coworkers [20,21] who demonstrated that IFN-γ exposure

increased Fas/CD95 expression and sensitized NB tumor cells to apoptosis, an effect that was further enhanced in the presence of *MYCN* amplification [20,21]. We conclude that additional investigation is warranted to evaluate the clinical utility of sequential administration of IFN-γ, doxorubicin and potentially even 5-aza-2′-deoxycytidine or other chemotherapeutic drugs to treat pediatric patients with refractory, late-stage neuroblastoma.

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